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# Tetrazole as a Replacement of the Electrophilic Group in Characteristic Prolyl Oligopeptidase Inhibitors

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**KEYWORDS:** *Prolyl oligopeptidase, inhibitor, synthesis, tetrazole, alpha-synuclein aggregation, molecular docking*

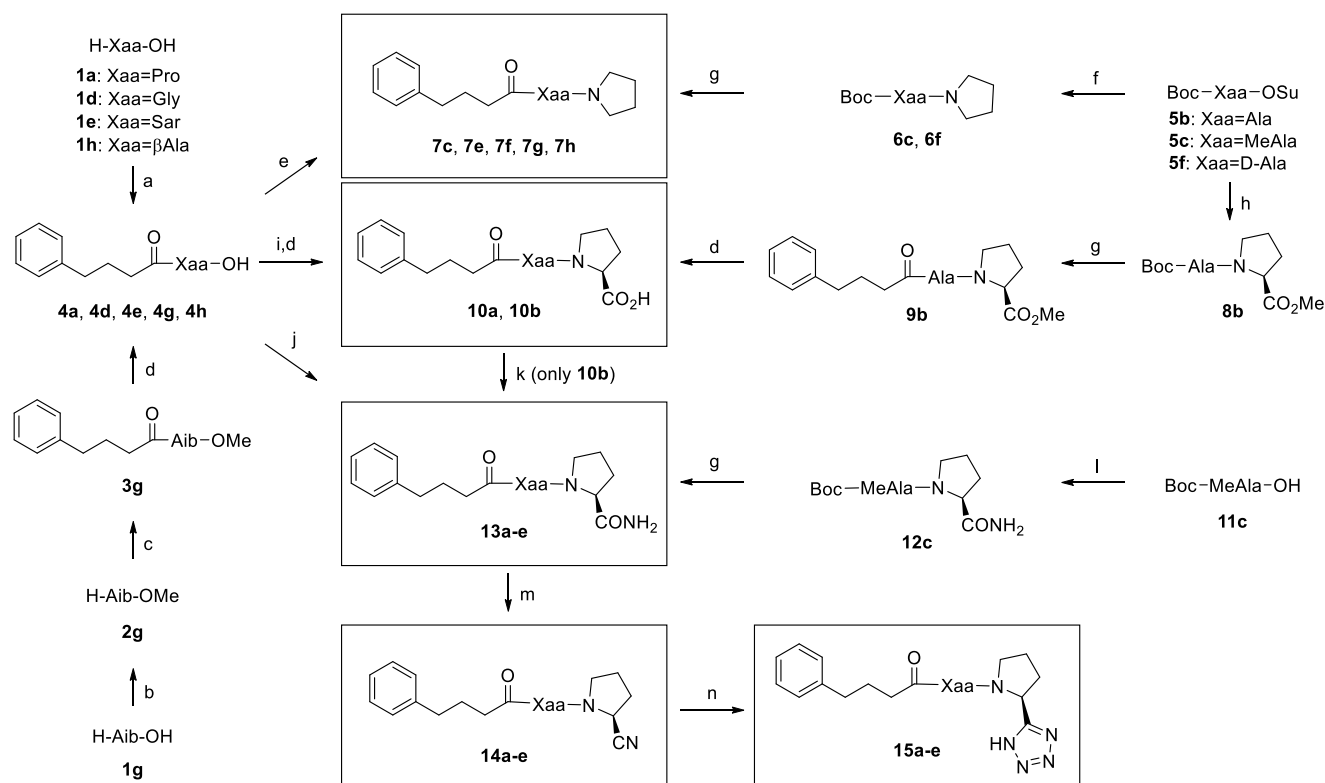
**ABSTRACT:** 4-Phenylbutanoyl-aminoacyl-2(*S*)-tetrazolylpyrrolidines were studied as prolyl oligopeptidase inhibitors. The compounds were more potent than expected from the assumption that the tetrazole would also here be a bioisostere of the carboxylic acid group and the corresponding carboxylic acids are at their best only weak inhibitors. The aminoacyl groups L-prolyl and L-alanyl gave potent inhibitors with IC<sub>50</sub> values of 12 and 129 nM, respectively. This was in line with typical prolyl oligopeptidase inhibitors, however, we did observe a difference with *N*-methyl-L-alanyl, which gave potent inhibitors in typical prolyl oligopeptidase inhibitors but not in our novel compound series. Furthermore, all studied 4-phenylbutanoyl-aminoacyl-2(*S*)-tetrazolylpyrrolidines decreased  $\alpha$ -synuclein dimerization at the concentration of 10  $\mu$ M, also when they were only weak inhibitors of the proteolytic activity of the enzyme with an IC<sub>50</sub> value of 205  $\mu$ M. Molecular docking studies revealed that the compounds are likely to bind differently to the enzyme compared to typical prolyl oligopeptidase inhibitors represented in this study by 4-phenylbutanoyl-aminoacyl-2(*S*)-cyano-pyrrolidines.

Prolyl oligopeptidase (PREP) is a serine protease with endopeptidase activity on short proline containing peptides cleaving the peptide on the carboxyl side of the proline residue, and many proline containing peptides, such as oxytocin and several neuropeptides, have been identified as its substrates *in vitro* (see for review Garcia-Horsman et al. 2007).<sup>1</sup> PREP is mainly found in the brain, more specifically in caudate nucleus and putamen, hippocampus, and cortex.<sup>2</sup> However, significant PREP activities and protein levels have been measured also in peripheral tissues such as skeletal muscle,<sup>3</sup> renal cortex, liver, ovaries, testis, and in ovarian and colorectal tumors.<sup>4–6</sup> Alterations in the enzyme activity of PREP have been observed in several diseases, including Parkinson's disease, Alzheimer's disease, Lewy body dementia,<sup>7</sup> and in several types of tumors.<sup>8,9</sup> Apart from its proteolytic activity, PREP has also other biologically important functions mediated by protein-protein interactions.<sup>10,11</sup> PREP is able to enhance dimerization of  $\alpha$ -synuclein ( $\alpha$ Syn), the key player in cellular toxicity in Parkinson's disease, via a direct protein-protein interaction.<sup>12</sup> PREP has highly flexible regions that are in equilibrium between many conformations and inhibitor binding stabilizes these flexible regions into one conformation.<sup>13</sup> Inhibited PREP has been shown to increase autophagy which is known to enhance clearance of aggregated forms of proteins and decrease dimerization of  $\alpha$ Syn *in vitro* and *in vivo*.<sup>14,15</sup>

Almost all known highly potent PREP inhibitors are structurally related to or recognizable mimetics of the peptide substrates with distinct P1, P2 and P3 sites.<sup>16</sup> The P1 moiety is mimicking the important L-proline (Pro) residue in the substrate and it is

typically a pyrrolidine with or without an electrophilic group in its 2-position. However, also five-membered heteroaryls such as 2-thienyl and 2-furanyl in the P1-position have resulted in potent inhibitors.<sup>17</sup> The P2 moiety can be a wide variety of aminoacyl groups or aminoacyl mimetics with Pro being the most preferred natural aminoacyl group, and the P3 moiety is typically a lipophilic acyl group. There are many examples of variations among the published inhibitors<sup>16</sup>, however, the two carbonyl groups of the connecting amide bonds have been difficult to replace and only few inhibitors lack one or both of them in their structures.<sup>18–21</sup>

The design for the new compounds came from one compound synthesized by the last author of this paper in his earlier research in the search for different replacements for the electrophilic group in PREP inhibitors, where the tetrazolyl group gave a surprisingly potent compound (unpublished data). In the present study, our primary aim was to investigate if 4-phenylbutanoyl-aminoacyl-2(*S*)-tetrazolylpyrrolidines as PREP inhibitors had the same structure-activity relationship as typical PREP inhibitor compound series such as 4-phenylbutanoyl-aminoacyl-pyrrolidines and 4-phenylbutanoyl-aminoacyl-2(*S*)-cyanopyrrolidines. A further aim was to study what effect the novel compound series had on protein-protein-mediated functions of PREP, such as catalysis of  $\alpha$ Syn dimerization. We were intrigued by the fact that the tetrazole ring is quite different from typical 2-substituents on the P1 pyrrolidine ring, especially its polar character is exceptional.



**Scheme 1.** Synthesis of the compounds, where a specific aminoacyl group is always with the same lower case letter in compound numbering. Reagents and conditions (a) 1. 10% aqueous Na<sub>2</sub>CO<sub>3</sub>, 4-phenylbutanoyl chloride / Et<sub>2</sub>O; (b) SOCl<sub>2</sub> / MeOH, reflux; (c) 4-phenylbutanoyl chloride, DIPEA / DCM; (d) LiOH / water, MeOH; (e) 1. pivaloyl chloride (or in its place ethyl chloroformate for **4g**), Et<sub>3</sub>N / DCM, 0 °C, 2. pyrrolidine, Et<sub>3</sub>N / DCM; (f) pyrrolidine / DCM; (g) 1. TFA / DCM, 0 °C, 2. 4-phenylbutanoyl chloride, Et<sub>3</sub>N / DCM (or alternatively for **6f**, 2. 1 M NaOH, 4-phenylbutanoyl chloride, / Et<sub>2</sub>O); (h) L-proline methyl ester, DIPEA / DCM; (i) 1. ethyl chloroformate, Et<sub>3</sub>N / DCM, 0 °C, 2. L-proline methyl ester, Et<sub>3</sub>N / DCM; (j) 1. pivaloyl chloride, Et<sub>3</sub>N / DCM, 0 °C, 2. L-prolinamide, Et<sub>3</sub>N / DCM; (k) 1. ethyl chloroformate, Et<sub>3</sub>N / THF, -10 °C, 2. NH<sub>3</sub> (7 M in MeOH); (l) 1. ethyl chloroformate, Et<sub>3</sub>N / DCM, 0 °C, 2. L-prolinamide, Et<sub>3</sub>N / DCM; (m) TFAA, Et<sub>3</sub>N / THF; (n) NaN<sub>3</sub>, NH<sub>4</sub>Cl / DMF, 100 °C.

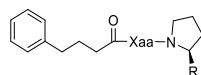
There are PREP inhibitors with five-membered heteroaromatics, which look slightly similar to our tetrazoles.<sup>22,23</sup> However, all of these have an electrophilic carbonyl group in exactly the same position as the typical electrophile. In addition, the tetrazole ring is negatively charged at physiological pH unlike the five-membered heteroaromatics in the published PREP inhibitors.

The synthetic routes for accessing the compounds in this study are presented in **Scheme 1**. Compounds **7a** (SUAM-1221), **7b** and **14a** (KYP-2047) were obtained from our old compound library at the University of Eastern Finland (**14a** was also synthesized here only as a synthetic intermediate on the route to **15a** but not used in the testing). The connecting amide bonds were synthesized using activation of carboxylic acid groups to corresponding acid chlorides or mixed anhydrides. These activation methods could not be used for non-N-alkylated chiral amino acids due to their susceptibility for racemization, and a milder activation to hydroxysuccinimide esters was used for them. Furthermore, in the case of 2-aminoisobutyric acid (Aib) the low solubility of the amino acid in the solvent of the first step of the synthetic route had to be circumvented, and in the case of *N*-methyl-L-alanine (MeAla) the slightly modified synthetic route gave improved yields and more easily separable

intermediates. The 4-phenylbutanoyl-aminoacyl-L-prolinamides were converted to the corresponding nitriles by dehydration with trifluoroacetic anhydride, and then further to the corresponding tetrazoles with sodium azide.

The inhibition of the proteolytic activity of PREP (IC<sub>50</sub>) for the compounds is presented in **Table 1**. The IC<sub>50</sub> values were in the beginning of the project determined using mouse brain homogenate, but we changed later to purified recombinant porcine PREP. Most unsubstituted pyrrolidines and prolinamides were tested with brain homogenate and all nitriles and tetrazoles were tested with purified porcine PREP. The IC<sub>50</sub> values for a few compounds were determined with both homogenate and pure enzyme to verify that the values were comparable.

In the beginning of the project earlier uncharted aminoacyl groups, Aib, sarcosine (Sar), MeAla and β-alanine (βAla) were evaluated at the P2 site in 4-phenylbutanoyl-aminoacyl-pyrrolidines, which is the most typical PREP inhibitor structure, and compared to the two natural aminoacyl groups Pro and L-alanine (Ala) which have been reported to give the most potent compounds.<sup>24</sup> In this study we did not carry out comparisons to L-phenylalanine, L-methionine, L-leucine, L-valine and glycine (Gly) as they have been previously shown to strongly decrease the inhibitory activity.<sup>24</sup>

**Table 1.** Inhibitory activities of the synthesized compounds.

Compound	Xaa	R	IC <sub>50</sub> (nM) enzyme	IC <sub>50</sub> 95% CI	IC <sub>50</sub> (nM) homogenate	IC <sub>50</sub> 95% CI
<b>7a (SUAM-1221)</b>	Pro	H	12	10-15	-	-
<b>7b</b>	Ala	H	147	115-191	-	-
<b>7c</b>	MeAla	H	-	-	298	142-571
<b>7e</b>	Sar	H	-	-	5141	3950-6604
<b>7f</b>	D-Ala	H	-	-	35180	12600-105300
<b>7g</b>	Aib	H	-	-	13510	8752-22570
<b>7h</b>	βAla	H	-	-	12080	7912-17790
<b>13a</b>	Pro	CONH <sub>2</sub>	4371	3841-4965	-	-
<b>13b</b>	Ala	CONH <sub>2</sub>	-	-	6213	3325-11340
<b>13c</b>	MeAla	CONH <sub>2</sub>	-	-	28 800	9792-97350
<b>13d</b>	Gly	CONH <sub>2</sub>	-	-	153 800	95 170-249 800
<b>13e</b>	Sar	CONH <sub>2</sub>	-	-	457 000	329 600-637 700
<b>14a (KYP-2047)</b>	Pro	CN	0.86	- <sup>a</sup>	0.3	- <sup>a</sup>
<b>14b</b>	Ala	CN	4.06	2.0-7.7	3.28	2.06-5.22
<b>14c</b>	MeAla	CN	5.4	4.5-6.4	-	-
<b>14d</b>	Gly	CN	220	117-454	264	138-493
<b>14e</b>	Sar	CN	269	- <sup>b</sup>	-	-
<b>15a</b>	Pro	tetrazolyl	12	9.9-14.7	-	-
<b>15b</b>	Ala	tetrazolyl	129	71-225	91	55-146
<b>15c</b>	MeAla	tetrazolyl	27 180	22 150-33 410	-	-
<b>15d</b>	Gly	tetrazolyl	205 400	- <sup>c</sup>	-	-
<b>15e</b>	Sar	tetrazolyl	10 640	8110-14 390	-	-
<b>10a</b>	Pro	CO <sub>2</sub> H	3626	2821-4965	-	-
<b>10b</b>	Ala	CO <sub>2</sub> H	17 540	14 100-22 100	-	-

<sup>a</sup> Confidence interval could not be determined since IC<sub>50</sub> value is lower than lowest used concentration.

<sup>b</sup> Confidence interval could not be determined since Hill slope is greater than -2.

<sup>c</sup> Confidence interval could not be determined since IC<sub>50</sub> value is too high.

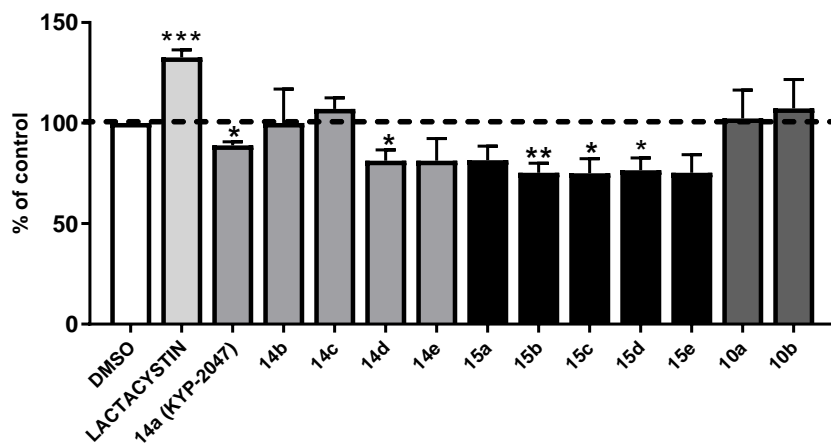
All explored aminoacyl groups were in fact one methyl/methylene group changes from Ala and of these only MeAla gave a potent compound **7c**. The other aminoacyl groups Sar, D-Ala, Aib and βAla gave compounds **7e**, **7f**, **7g** and **7h**, respectively, with significantly reduced inhibitory activities.

Pro, Ala, MeAla, Gly and Sar were selected as the aminoacyl groups for our novel tetrazoles. They were synthesized via the corresponding prolinamide and nitrile intermediates, which were also tested for their inhibitory activities. As expected, the prolinamide intermediates **13a-13e** had only weak inhibitory activities and the nitrile intermediates **14a-14e** were highly potent inhibitors. The electrophilic nitrile group is known to have favorable interaction with the catalytic Ser554 residue at the active site of the enzyme, and for **14a** (which is the well-known reference compound KYP-2047)<sup>25</sup> this has been described as a reversible covalent interaction.<sup>26,27</sup> For the nitriles, also Pro, Ala and MeAla gave the most potent inhibitors **14a**, **14b** and **14c**, with IC<sub>50</sub> values of 0.86, 4.06 and 5.4 nM, respectively. These IC<sub>50</sub> values should be used with caution as at least **14a** is a covalently binding compound with slow, tight binding enzyme kinetics.

Among our target tetrazoles **15a-15e**, Pro and Ala gave the most potent inhibitors **15a** and **15b**, with IC<sub>50</sub> values of 12 and

129 nM, respectively. Surprisingly, MeAla gave only a weak inhibitor **15c**, which was over 200-fold lower in inhibitory activity than **15b**. It is obvious that the N-methyl group on Ala is not favorable in combination with the tetrazole group. Gly and Sar gave the tetrazoles **15d** and **15e**, which had weak inhibitory activities of 205 and 10.6 μM, respectively. The carboxylic acid analogues **10a** and **10b** of the most potent tetrazoles **15a** and **15b**, respectively, had only weak inhibitory activities in the micromolar range, indicating no bioisosterism between the tetrazole and the carboxylic acid group in this series of compounds.

For evaluating the ability of the tetrazoles **15a-15e** to reduce αSyn dimerization we used a slightly modified PCA (protein fragment complementation assay) method described in Savolainen et al. 2015.<sup>12</sup> We used mouse N2A cells in the assay which has high endogenous PREP activity (Suppl. Fig 1.). Results of the assay are shown in Figure 1. Interestingly, all tetrazoles **15a-e** decreased αSyn dimerization. The αSyn dimerization assay was performed at the concentration of 10 μM and even tetrazole **15d** with an IC<sub>50</sub> value of 205 μM reduced αSyn dimerization with the same magnitude as tetrazoles **15a** and **15b** with IC<sub>50</sub> values 12 and 129 nM, respectively.



**Figure 1.** Results of the  $\alpha$ Syn- $\alpha$ Syn2 dimerization in PCA (protein fragment complementation assay) with 4 h treatment and 10  $\mu$ M concentration. Tetrazoles **15a-e** reduced the amount of  $\alpha$ Syn dimers 18-25% compared to control. **14a** (KYP-2047), **14d** and **14e** were the only compounds from nitrile group which were able to reduce  $\alpha$ Syn dimerization. Carboxyl acids **10a** and **10b** were not able to reduce formation of  $\alpha$ Syn dimers. Lactacystin serves as positive control for  $\alpha$ Syn dimerization. Data are presented as means+SEM ( $n \geq 3$ , \*\*\*  $p < 0.001$ , \*\* $p < 0.01$ , \* $p < 0.05$ )

We decided also to examine the nitrile intermediates as a comparison. The nitrile **14a** (KYP-2047) had been verified to reduced  $\alpha$ Syn dimerization in several earlier studies, but the other nitriles had not been studied.<sup>12,14</sup> To our surprise, the nitriles **14b** and **14c** with aminoacyl groups Ala and MeAla did not have an effect on  $\alpha$ Syn dimerization although they are 4-5 nM inhibitors of PREP, and on the other hand, the nitriles **14d** and **14e** with aminoacyl groups Gly and Sar had an effect although they are only 220-260 nM inhibitors of PREP. It is important to highlight here that only compounds **14a** (89%,  $p < 0.05$ ), **14d** (81%,  $p < 0.05$ ), **15b** (75%,  $p < 0.01$ ), **15c** (75%,  $p < 0.05$ ) and **15d** (77%,  $p < 0.05$ ) had statistically significant decrease in  $\alpha$ Syn dimerization (Student's t-test compared to DMSO control,  $n \geq 3$ ).

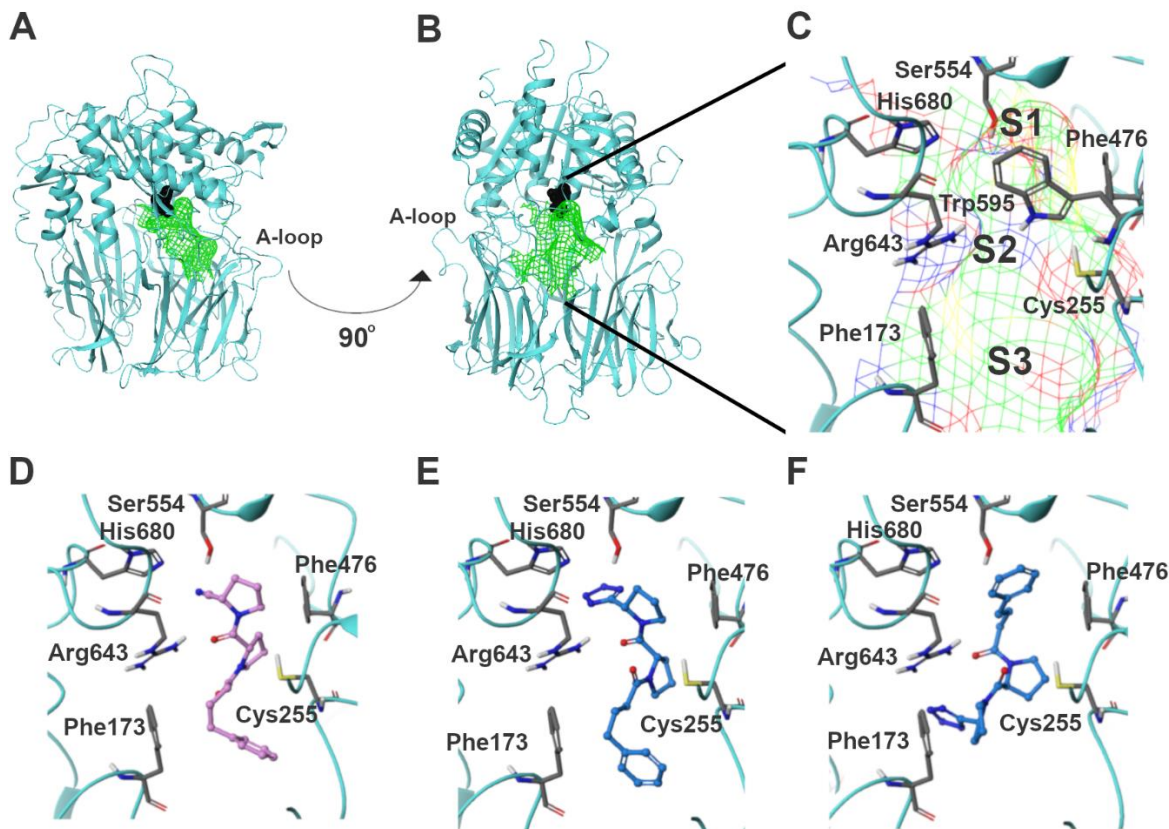
The  $\alpha$ Syn dimerization assay results for both nitriles and tetrazoles clearly indicate that the structure-activity relationship for affecting this function of PREP is slightly different from inhibiting the proteolytic activity. To study the binding of the nitriles **14a-e** and tetrazoles **15a-e** to PREP, molecular docking studies were performed (Figure 2A-F). The binding pocket included the commonly known S1, S2, and S3 subsites (Figure 2C). Among the nitriles, **14a** is known to bind covalently to the catalytically active serine residue (Ser554) at S1.<sup>28</sup> Other nitriles could be assumed to orient similarly to **14a** directing the nitrile group towards S1 and Ser554 (Figure 2D). Indeed, all nitriles could place the nitrile group at S1 and the phenyl group at S3. The most potent nitriles **14a**, **14b**, and **14c** directed the nitrile group towards Ser554. However, in docking studies **14d** and **14e** could not orient the nitrile group towards Ser554. This could maybe explain why they are less potent inhibitors than the other nitriles. In the docking protocol the covalent interaction between the nitrile group and Ser554 was not assessed. Thus, the possible covalent bond formation could actually force the nitrile groups to S1.

Interestingly, none of the tetrazoles formed an interaction to Ser554 even though they could place the tetrazole ring at S1. The poses with the tetrazole ring at S1 were compared to the poses of the corresponding nitriles with the nitrile group at S1 (Suppl. Fig. 2-6). The comparison revealed that the tetrazole

ring might be positioned in the binding pocket slightly differently than the nitrile group. This can be seen most clearly between compounds **14a** and **15a**, and compounds **14d** and **15d**. The two most potent tetrazoles **15a** and **15b** were inclined to form an interaction between their negatively charged tetrazole group to the positively charged Arg643 instead of Ser554 (Suppl. Fig. 7 and 8). For the other tetrazoles the tetrazole ring at S1 was not forming any interactions with amino acid residues in the binding pocket (Suppl. Fig. 9-11). Moreover, the pose of **15e** was tilted when compared to other tetrazoles or nitriles, and its phenyl group positioned outside the pocket (Suppl. Fig. 11.).

The docking results proposed a putative binding pose in which all the tetrazoles could fit to the binding pocket. In this hypothetical pose the phenyl group was at S1 instead of S3 (Suppl. Fig. 12-16). Tetrazoles might prefer to position the lipophilic benzene ring rather than the hydrophilic tetrazole ring into the hydrophobic S1 pocket. The tetrazole ring does not have a similar ability as the nitrile group to form a covalent bond to Ser554, which is an important interaction for at least some nitriles in anchoring them at S1. As a less hydrophilic moiety, the nitrile group might also be more easily placed into the S1 pocket than the tetrazole ring. Overall, these results suggest that the tetrazoles might have two putative binding poses: the phenyl group at S3 (Figure 2E) or at S1 (Figure 2F).

In conclusion, although the tetrazole group is a known common bioisostere for the carboxylic acid group, the tetrazole is clearly not a bioisostere of a carboxylic acid group in PREP inhibitors. The tetrazoles were more potent inhibitors of the proteolytic activity and  $\alpha$ Syn dimerization catalyzing effect of PREP than the corresponding carboxylic acids. The present study further hypothesized that the tetrazoles might have another binding mode in addition to the known binding mode of PREP inhibitors. In this putative binding mode, the tetrazole ring is placed at S3 and the phenyl group at S1. Remarkably, all tetrazoles reduced  $\alpha$ Syn dimerization despite the fact that some of them only were weak inhibitors of the proteolytic activity. These findings taken together possibly also indicate that there could be another alternative binding site in enzyme, which affects more the  $\alpha$ Syn dimerization catalyzing effect than the proteolytic activity of the enzyme.



**Figure 2.** Putative binding site of the tetrazoles with PREP. (A) The crystal structure of PREP. The catalytically active serine residue (Ser554) is marked with black, and the inhibitor-binding site is marked with green mesh. (B) The crystal structure of PREP from site of Figures C-F. (C) The ligand-binding pocket with the S1, S2, and S3 subsites. Green indicates lipophilic, yellow aromatic, red electronegative, and blue electropositive areas. (D) Compound **14a** at the inhibitor-binding site. The nitrile points towards Ser554 and forms hydrogen bond to it (not shown in the figure). (E) Compound **15a** at the inhibitor-binding site in the commonly known binding mode. (F) A suggested hypothetical binding mode for the tetrazoles with compound **15a** as a representative compound.

## EXPERIMENTAL SECTION

**Synthesis and characterization of novel compounds.** Synthesis and characterization of all compounds are reported in detail in supporting information.

**Expression and purification of recombinant porcine PREP.** Porcine PREP enzyme was expressed and purified according protocol described by Venäläinen et al. 2002.<sup>29</sup>

**Determination of IC<sub>50</sub> value.** Preparation of mice brain homogenates and detailed procedure for in vitro assay for inhibitory activity are found in supporting information.

**PCA for αSyn dimerization.** Detailed cell culture, procedure and used DNA constructs are found in supporting information.

**Molecular docking studies.** Protocol is described in supporting information.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

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### Author Contributions

The manuscript was written through contributions of all authors. Chemistry was contributed by TPK and EAAW, pharmacology by TPK, TSE and TTM, and molecular docking studies by JKT and MKLK. All authors have given approval to the final version of the manuscript.

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## ABBREVIATIONS

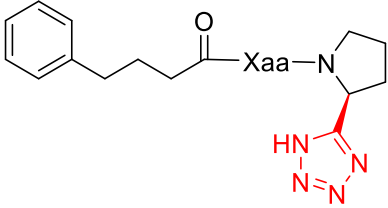
PREP, prolyl oligopeptidase;  $\alpha$ Syn, alpha-synuclein; Aib, 2-aminoisobutyric acid; Sar, sarcosine; MeAla, *N*-methyl-L-alanine;  $\beta$ Ala, beta-alanine; PCA, protein fragment complementation assay.

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Tetrazole as a Replacement of the Electrophilic Group in Characteristic Prolyl Oligopeptidase Inhibitors

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	Xaa	IC <sub>50</sub> (nM)	Effect on $\alpha$ Syn dimeriz. at 10 $\mu$ M
	Pro	12	82 %
	Ala	129	75 %
	Sar	10 640	75 %
	MeAla	27 180	75 %
	Gly	205 400	77 %

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